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The alternative sigma factor σ^L of *L. monocytogenes* promotes growth under diverse environmental stresses

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The alternative sigma factor σ^L of *L. monocytogenes* promotes growth under diverse environmental stresses

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1. Summary

Listeria monocytogenes L.m. are important food-borne pathogens that can cause outbreaks of serious human disease. These organisms frequently colonize and proliferate on preserved food products despite exposure to stress conditions induced by low storage temperatures, inclusion of organic acid based preservatives and high osmolarity. To assess alternative sigma factor σ^L contributions to such stress resistance of *L. m.*, quantitative RT-PCR assays and *sigL* gene deletion mutagenesis were applied in *L. m.* EGDe. Transcription of *sigL* was significantly induced by growth of EGDe under cold, organic acid and elevated NaCl salt concentration stress conditions. The growth of a Δ*sigL* strain exposed to these stress conditions was also found to be significantly impaired in comparison to that of its isogenic wild-type strain. The contribution of σ^L to transcription control of cold and NaCl stress adaptation genes, *oppA*, *cspD* and *clpP* was also comparatively assessed in Δ*sigL* and wild type EGDe cells. Transcription of the *oppA* gene, which encodes the OppA protein that also promotes *L. m.* cold growth, was significantly reduced in cold stress grown Δ*sigL* cells compared to levels of the wild type EGDe strain. These findings therefore suggest important roles of σ^L regulatory pathways in facilitating resistance of *L.m.* organisms against stress conditions associated with low storage temperatures, exposure to organic acid and elevated NaCl salt concentrations.

Keywords: *Listeria monocytogenes*, Stress adaptation, Alternative sigma factor, Sigma L, Gene expression

2. Introduction

Listeria monocytogenes is a gram-positive food-borne pathogen of public health concern as well as a considerable food safety challenge. Various strains of these organisms have been associated with outbreaks of severe human disease leading to relatively high mortality in different parts of the world (Ramaswamy *et al.*, 2007; Swaminathan & Gerner-Smidt, 2007). The ubiquity of these organisms in nature and in a wide range of food related environments presents a considerable microbial control challenge in food production. In particular, these organisms survive and proliferate on ready to eat (RTE) foods stored at low temperatures under conditions of high NaCl salt concentration and in presence of organic acid based food preservatives (Lianou & Sofos, 2007; Nufer *et al.*, 2007; Pal *et al.*, 2008). The nature of physiological mechanisms leading to such cellular stress resistance phenotypes in these food-borne pathogens is still not understood. The alternative sigma factor protein systems are central to transcription mechanisms that control the function and specificity of bacterial RNA polymerase (RNAP) holoenzyme complexes (Chaturongakul *et al.*, 2008). These proteins provide key regulatory points for modulating different cellular functions required to respond to constantly changing environmental stress conditions encountered by *L. monocytogenes* microorganisms. The existence of at least five different alternative sigma factor proteins has been documented in *L. monocytogenes* (Glaser *et al.*, 2001; Metzger *et al.*, 1994; Zhang *et al.*, 2005). These are σ^A (RpoD), σ^B, σ^C and σ^H proteins, which all belong to the σ^{70} family, as well as σ^L (RpoN), representing the only σ^{54} family member in this bacterium. The σ^A protein also known as RpoD, has been proposed as the primary sigma factor responsible for regulating house keeping gene expressions (Metzger *et al.*, 1994). The σ^B protein is involved in general stress responses and some virulence functions of this organism (Chaturongakul *et al.*, 2008). As examples, this sigma

factor protein promotes adaptation to low pH, high osmolarity, oxidative stress and low temperature stress conditions in this bacterium (Becker *et al.*, 1998; Becker *et al.*, 2000; Ferreira *et al.*, 2001; Moorhead & Dykes, 2003). Alternative sigma factor σ^H functions are less defined, although this protein was found to be induced by low pH, and the growth of a *sigH* deletion mutant was impaired in minimal media, as well as under alkaline stress conditions (Phan-Thanh & Mahouin, 1999; Rea *et al.*, 2004). Alternative sigma factor σ^C has so far only been described in genetic lineage II *L. monocytogenes* strains, which predominate in food associated environmental niches (Chen *et al.*, 2006; Gray *et al.*, 2004; Zhang *et al.*, 2005). *L. monocytogenes* σ^C originally described as a lineage II specific regulator of thermal adaptation and resistance mechanisms, was also recently linked to regulation of cold stress adaptation responses in this organism (Chan *et al.*, 2008; Zhang *et al.*, 2005).

The bacterial σ^{54} class of sigma factors includes important regulators of metabolic functions and virulence properties in different species of bacteria (Buck *et al.*, 2000). The roles of the σ^L alternative sigma factor protein in *L. monocytogenes* are not yet fully understood. Its functions in this organism will be of particular interest given its distinctive functional mode. σ^L additionally needs to bind an activator protein in order to melt target promoter DNA sequences and activate transcription. This is in contrast to the classical σ^{70} sigma factors that can directly melt target promoter DNA sequences and activate transcription. Moreover, the σ^L -RNAP holoenzyme transcription complex activates target genes proceeded by -24/-12 promoters, whilst σ^{70} controlled RNAP holoenzyme complexes targets are proceeded by -35/-10 promoters (Buck *et al.*, 2000).

A global transcription analysis of a *sigL* deletion mutant in *L. monocytogenes* revealed that at least seventy-seven σ^L regulated genes exist in this bacterium (Arous *et al.*, 2004a). Although most of σ^L controlled genes were mainly related to

carbohydrate and amino acid metabolism, the σ^L regulon also includes genes involved in other cellular pathways and stress adaptation responses. σ^L might also play an important role in intracellular stress adaptation of *L. monocytogenes*. A transcription profiling study found that several σ^L regulated genes also get up-regulated during intracellular growth of *L. monocytogenes* in a murine macrophage model (Chatterjee *et al.*, 2006). *L. monocytogenes sigL* deletion mutants that are compromised in osmotic stress tolerance or displaying increased resistance to class II bacteriocin mesentericin Y105 have been described (Okada *et al.*, 2006; Robichon *et al.*, 1997). The resistance of *L. monocytogenes* to class II bacteriocins has been linked to repression or lack of gene expression from the directly σ^L controlled *mptACD* operon, which encodes the EII_t^{Man} mannose PTS permease (Arous *et al.*, 2004b).

The current study has used quantitative gene expression analysis and phenotypic characterization of a *sigL* deletion mutant in order to explore **σ^L** contribution to *L. monocytogenes* adaptation to different stress conditions associated with RTE food preservation measures.

3. Material and Methods

3.1. Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* XL-1 Blue strain host was employed for plasmid construction and propagation steps (Bullock *et al.*, 1987). Bacterial strains were routinely grown in Luria-Bertani (LB) medium (Difco Laboratories) and brain heart infusion broth (BHI) (Oxoid). Agar was added at 1.5% for solid media. Defined minimal medium (DM) was prepared as previously described (Premaratne *et al.*, 1991). The NaCl stress conditions were created in DM and BHI broths by addition of 2.2% (376 mM) and 3% (599 mM) NaCl, giving rise to the DMS and BHI-NaCl broths, respectively. Organic acid based stress conditions were created by adjusting BHI from pH 7.4 to pH 6.0 using a 3M lactic acid solution. Ampicillin and chloramphenicol were used for selection at 50 µg/ml and 10 µg/ml, respectively. *E. coli* and *L. monocytogenes* cells were made electrocompetent as previously described (Sheng *et al.*, 1995; Monk *et al.*, 2008).

3.2. DNA manipulation

Genomic bacterial DNA was isolated using the DNeasy blood and tissue kit (Qiagen), and Plasmid DNA with the Plasmid Midi kit (Qiagen), according to the procedures provided in the respective kit protocols. The FastStart High Fidelity PCR System (Roche Molecular Diagnostics) was used for PCR amplifications based on the kit suppliers' protocols. DNA restriction enzymes (Roche Molecular Diagnostics) and T4 DNA ligase (New England Biolabs) were also applied as per suppliers' recommendations. PCR products and restriction enzyme digested DNA fragments were purified from agarose gels following the Mini Elute gel extraction kit protocols (Qiagen). Oligonucleotide primers were ordered from Microsynth AG (Balgach, Switzerland), who also provided the DNA sequencing services .

3.3. Construction of the Δ*sigL* strain

The pKSV7 plasmid (Smith & Youngman, 1992) and the splicing-by-overlap extension (SOE) PCR method (Horton *et al.*, 1990) were used to create an in-frame deletion of the *sigL* gene in the *L. monocytogenes* EGDe. A DNA fragment carrying a *sigL* gene deletion was created by amplification of the EGDe genomic DNA template using SOE primers listed in Table 1. The Δ*sigL* DNA fragment was subsequently cloned into the pKSV7 plasmid creating the pKSV*7*-Δ*sigL* construct. This was transformed into electrocompetent *L. monocytogenes* EGDe cells and correct transformants were isolated as previously described (Schmid et al., 2009). The deletion of the targeted *sigL* gene region in *L. monocytogenes* EGDe cells was facilitated by homologous recombination following a previously described experimental protocol (Schmid et al., 2009). The *sigL*-deleted mutant was identified by PCR amplification that targeted the EGDe DNA region that spanned the *sigL* gene locus. DNA sequencing of the amplified deleted region confirmed the mutant strain.

3.4. Growth curves

Stationary phase (10^9 CFU/ml) inocula of each strain were prepared from single colonies, which were grown overnight (16-18 hrs) in 10 ml of BHI (37℃, shaking at 220 rpm). The stationary phase cultures grown in this manner were 10-fold serially diluted in 0.85% NaCl-peptone solution to 10^5CFU/ml. 10 ml of BHI (pH 7.4), acidified BHI (pH 6.0), BHI-NaCl, DM and DMS were inoculated at 10^3 CFU/ml using stationary phase inoculums prepared in this way. Inoculated cultures were incubated without shaking at 37, 10 and 4℃. The growth of each sample was monitored by standard colony counting methods at defined time intervals.

3.5. Stress exposure

To characterize the effect of NaCl salt, acid and cold stress exposure on *sigL* gene expression, stationary phase EGDe cultures were also prepared as described above. 30 ml of BHI (pH 7.4), acidified BHI (pH 6.0) and BHI-NaCl were inoculated at 10^6CFU/ml. All samples were incubated without shaking at 37℃, except for the cold stress grown samples, which were incubated at 10℃. Growth of each sample was similarly followed by colony counting. Cultures in mid exponential growth stage (10^8 CFU/ml) were in each case collected and processed for total RNA template isolation as described below. To monitor σ^L dependent target gene expression under cold and NaCl salt stress conditions, mid logarithmic EGDe wild type and Δ*sigL* cultures were similarly prepared in BHI-NaCl (37℃) and regular BHI (10℃) cultures and also processed to isolate respective total RNA templates from the samples.

3.6. RNA isolation and reverse transcription

A total RNA isolation protocol that included mechanical lysis of *L. monocytogenes* cells and column based RNA purification was applied. 1.5 ml aliquots of samples prepared as described above were centrifuged for 5 minutes at 4000g. This step was performed at 10℃ for the cold stress grown samples, and room temperature for the rest of the samples. The resulting cell pellets were resuspended in 0.5 ml lysis buffer of the RNeasy Plus Mini Kit (Qiagen). The mixtures were transferred onto the lysing bead matrix in MagNA lyser tubes (Roche Molecular Diagnostics GmbH, Penzburg, Germany), and mechanically disrupted using the MagNA Lyser Instrument (6500 rpm for 60 secs). Total RNA was purified from the lysates according to the RNeasy Plus Mini Kit protocol. Two DNA removal steps were applied. First the samples were passed over genomic DNA removal columns and then an on-column DNAse I digestion step was performed after binding to the RNA

purification column. Total RNA was eluted in 30 µl of RNAse-free water and the yield determined by measuring absorption at 260 nm in the Nanodrop ND1000 (Nanodrop instruments, Delaware, USA). The reverse transcription step was performed using the Quantitect Reverse Transcription Kit (Qiagen), which incorporates an optimized blend of oligo-dT and random hexamers for priming cDNA synthesis. 300 ng of total RNA from each sample were converted into cDNA in 20 µl reactions. As controls similar amounts of total RNA of each sample were also subjected to the cDNA synthesis reaction without the reverse transcriptase enzyme. These provided the minus RT control samples used assess potential residual DNA contamination of each sample in the gene specific real-time PCR reactions.

3.7. Quantitative real-time PCR analysis

The different target genes and primers of this study are also listed in Table 1. The Light Cycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland) was used to determine the relative quantitative gene expression levels. PCR primers to amplify each target were designed using the LC probe design software (Roche Molecular Diagnostics). Primers targeting each gene and their concentrations (200-500 nM) were optimized to achieve specific and efficient target amplification (90 -100% PCR efficiency). *L. monocytogenes* EGDe genomic DNA based standard curves were used to determine PCR amplification efficiency of each primer pair. Real-time PCR reactions were performed in 10 µl using 4ng of the cDNA template pool (2.5 µl of a 1:10 diluted reverse transcription reaction). The rest of the reaction mixture contained 5 µl of 2XLightCycler^{R} 480 SYBR Green I master mix (Roche Molecular Diagnostics, Germany) and 2.5 µl of the forward and reverse mixture. As negative controls, water and the no RT control reaction of each sample were included as PCR templates. The real-time PCR run protocol consisted of

i) preincubation (4 min at 95°C); ii) amplification (10s at 95°C; 20s at 56°C; 20s at 72°C; 5s at 80°C with a single fluorescent measurement); melting curve (65-97°C at 2.2°C/s and a continuous fluorescent measurement) and iv) cooling. Target gene transcript levels were determined using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). An initial reference gene validation under experimental conditions of this study was performed as previously described to determine the most suitable gene for relative mRNA quantification normalization (Tasara and Stephan 2006). The *16S rRNA* gene was determined to be the most suitable internal control reference based on the stability of its expression under experimental conditions our study.

3.8. Statistical analysis

The statistical analysis in this study was performed using the Stat View 4.02 (Abacus Concepts Inc., Berkeley, California, USA) program. The bacterial growth colony counts were converted into log CFU/ml. In the case of quantitative RT-PCR, target gene mRNA transcripts levels quantified relative to *16S rRNA* levels were log-transformed (log10) to normalize the data. In all cases means and standard deviations were based on results from at least three independent experimental runs. The statistical significance of differences in growth and gene expression data reported in this study was determined based on Students t-test analysis. Differences with P values <0.05 were considered to be statistically significant.

4. Results

4.1. Cold, NaCl salt and acid stress induction of *sigL* gene expression

The influence on *sigL* gene expression of different stress conditions was quantitatively assessed by real-time RT-PCR in stress exposed mid exponential growth phase *L. monocytogenes* EGDe cells. As shown in figure 1, adaptation of *L. monocytogenes* adaptation to cold (BHI 10°C), NaCl salt (BHI plus 3% NaCl) and acid (BHI pH 6.0) stress conditions led to significant induction of *sigL* mRNA transcripts compared to the levels found in control EGDe cells that had been similarly grown in regular BHI at 37°C.

4.2. Growth of Δ*sigL* in BHI and DM at low temperatures

The growths of *L. monocytogenes* EGDe and its isogenic Δ*sigL* mutant strain was also evaluated in BHI and DM broth cultures incubated under optimal (37°C) and cold (10 and 4°C) stress temperature conditions. We found no significant differences in the growth behavior of the two strains in both media during incubation at 37°C (data not shown). Similarly the loss of *sigL* function had a limited impact on *L. monocytogenes* cold growth in BHI. As shown the growth of the Δ*sigL* mutant and wild-type EGDe strain were almost similar in BHI cultures held at 10 and 4°C (see Figures 2A and 4B). The growth of Δ*sigL* mutant was, however, significantly ($P<0.05$) impaired compared to EGDe under similar cold stress conditions in DM. As an example the Δ*sigL* mutant had a longer lag phase (188 vs. 19 hrs) and grew at a slower rate (0.023 vs. 0.038 log/hr) compared to the EGDe strain in DM cultures held at 10°C (Figure 2B). Similar poor growth of Δ*sigL* mutant was also observed in DM at 4°C (data not shown). The cold growth of *L. monocytogenes* may depend on presence of cryoprotective osmolytes such as betaine and carnitine. We therefore also examined if glycine betaine or carnitine supplementation could rescue the cold

growth defects associated with the Δ*sigL* mutant. Supplementation of each of these two substances significantly improved the cold growth capacity of the Δ*sigL* strain in DM at 10℃ (Figure 2C). Although the Δ*sigL* mutant was still unable to recover its cold growth capacity under these conditions to levels associated with wild type EGDe strain cultivated in regular DM cultures under similar cold stress conditions.

4.3. Growth of Δ*sigL* under NaCl stress

Next we also investigated the growth of EGDe and Δ*sigL* strains in presence of NaCl salt stress in DMS (DM plus 2.2% NaCl). The growth of the two strains in DMS cultures at 37℃ is presented in figure 3A. As shown growth of the Δ*sigL* mutant was impaired relative to that of wild-type EGDe strain under such stress conditions. Specifically, the Δ*sigL* mutant displayed an initial decrease of the inoculum, which was not observed in the wild-type EGDe strain exposed to same conditions. Growth resumption of the Δ*sigL* mutant occurred after more than 30 hours of lag phase compared to only 8 hours in the wild type strain. Growth of the Δ*sigL* mutant strain was also evaluated in DMS at 10℃ (Figure 3B). This revealed further growth impairment in this strain under such NaCl stress conditions at lower temperatures. In this case growth resumption was observed after a lag phase of about 10 days. This was in contrast 7.8 days of lag phase observed in DM cultures of the same strain that were also held at 10℃. Moreover the Δ*sigL* mutant achieved in DMS only one-log (10^3 to 10^4 CFU/ml) over 14 days compared to a three-log (10^3 to 10^7 CFU/ml) growth when similarly incubated DM.

4.4. Growth of Δ*sigL* in BHI under lactic acid stress

The influence of *sigL* deletion on *L. monocytogenes* growth under acid stress was also examined in BHI cultures that had been acidified to pH 6.0 using lactic acid.

No significant growth differences were observed between the Δ*sigL* mutant and the wild-type EGDe at 37℃ under such acid stress conditions (data not shown). In contrast Δ*sigL* mutant growth was significantly impaired under acid stress in samples held at 4℃ (Figure 4). Both the Δ*sigL* mutant and wild type EGDe strains grew from 10^3 CFU/ml to reach 10^8 CFU/ml over 5 weeks when incubated in cultures of regular BHI (pH 7.0) at 4℃ (Figure 4A). Under acid stress conditions, although the wild type EGDe strain grew over 8 weeks at 4℃ to reach 10^8 CFU/ml, the Δ*sigL* strain failed to grow under these conditions (Figure 4B).

4.5. Evaluation of σ^L regulated gene transcription under cold and NaCl stress conditions

The expression of σ^L controlled target genes with experimentally confirmed functional contributions to cold and osmotic stress adaptation was comparatively evaluated in stress adapted wild-type EGDe and Δ*sigL* strain cells. As a control, the transcript levels of *mptA*, a direct σ^L dependent *gene,* were also determined in these two strains. As expected *mptA* transcripts were found to be consistently lower in Δ*sigL* mutant cells compared to wild type EGDe cells in cold and NaCl stress adapted cells (Figures 5A and B). The expression of *cspD and oppA* transcripts is partly regulated through σ^L dependent pathways in *L. monocytogenes* (Arous et al., 2004). Both genes have also been implicated in promotion of cold growth in this bacterium (Borezee et al., 2000; Schmid et al., 2009). No significant ($P>0.05$) differences were detected in the level of *cspD* mRNA transcripts of cold stress grown EGDe and Δ*sigL* cells (Figure 5A). On the other hand *oppA* mRNA levels were significantly ($P<0.05$) lower (53-fold) in such cold stress grown Δ*sigL* cells than in wild-type EGDe (Figure 5A). Functional contribution of both *clP* and *cspD* in NaCl stress tolerance of *L. monocytogene*s has also been reported (Gaillot et al., 2000;

Schmid et al., 2009). However, there were no significant differences in *cspD* and *clpP* mRNA transcripts detected in NaCl salt stress grown cells between Δ*sigL* and EGDe (Figure 5B).

5. Discussion

The control of *L. monocytogenes* along the food supply chain remains a considerable challenge. The improved control of this pathogen in foods will also depend on understanding of the molecular response mechanisms associated with the stress resistance phenotypes of this bacterium. The alternative sigma factor σ^L proteins provide unique transcription regulation functions and may play an important role in promoting *L. monocytogenes* stress resistance in food environments. In the present study we have investigated σ^L roles in *L. monocytogenes* stress adaptation against environmental conditions encountered in RTE food preservation. We show that σ^L functions are important during *L. monocytogenes* growth in defined minimal media at low temperatures. The growth of a Δ*sigL* strain is impaired compared to the wild type EGDe strain in DM at both 10 and 4℃, and significant induction of *sigL* gene expression was observed in wild-type EGDe cells exposed to cold growth conditions in BHI at 10℃. We have also presented experimental evidence supporting the σ^L involvement in efficient osmotic stress resistance of *L. monocytogenes*. This was shown by both NaCl stress dependent induction of *sigL* gene expression, as well as an impaired growth phenotypes of the Δ*sigL* mutant under NaCl (2.2%) salt stress in DMS cultures held at different incubation temperatures. Finally, similar experimental evidence suggesting σ^L contribution to *L. monocytogenes* organic acid stress resistance has also been shown. Both *sigL* gene expression induction in wild type EGDe, and growth failure of Δ*sigL* mutant under acid stress exposure using lactic acid acidified BHI (pH 6.0) media was presented. The findings of this study are in agreement with previous experimental evidence of σ^L contribution to cold and NaCl stress adaptation reported in this organism (Chan *et al.*, 2008; Liu *et al.*, 2002; Okada *et al.*, 2006). Okada *et al.* (2006), described reduced osmotic stress tolerance of a Δ*sigL* EGDe mutant strain in comparison to the wild-type strain of this organism. In a

transcriptome profiling based study by Liu *et al.* (2002), they also found that *sigL* transcripts levels were higher during growth at 10°C compared to 37°C using the *L. monocytogenes* EGDe strain. Chan *et al.* (2008), recently reported that cold growth of an *L. monocytogenes* 10403S Δ*sigL* mutant strain was also slightly poorer than its isogenic wild-type strain in BHI at 4°C (Chan *et al.*, 2008). The σ^L contribution to organic acid stress tolerance of *L. monocytogenes* has however not yet been documented to our knowledge.

Detailed knowledge on how loss of σ^L function leads to cold stress sensitivity in *L. monocytogenes* organisms is currently lacking. The loss of σ^L dependent pathways through deletion of *sigL* or its *bkdR* and *yplP* transcription enhancers, similarly induces cold stress sensitive phenotypes in the gram positive model organism, *Bacillus subtilis* (Wiegeshoff *et al.*, 2006). It is presumed that the *bkd* operon expression is activated in response to cold stress through a σ^L controlled transcription complex that also contains the BkdR enhancer protein (Wiegeshoff *et al.*, 2006). Loss of *sigL* or *bkdR* functions thus prevents σ^L dependent induction of the *bkd* operon in response to cold stress. This indirectly hinders *de novo* branched fatty acid chain (BCFA) synthesis, which requires the conversion of isoleucine to a-keto acids. As a consequence cold exposed cells of a *B. subtilis* Δ*sigL* mutant are presumably impaired in their capacity to adjust their membrane lipid composition at low temperatures in order to maintain flexibility and function of membrane structures. Although molecular pathways linking σ^L function to *bkd* operon transcription control have not yet been shown in *L. monocytogenes*, the disruption of *bkd* operon genes also impairs cold growth and leads to altered membrane fatty acid composition in cells of this bacterium (Zhu *et al.*, 2005).

Stress sensitivity phenotypes observed in the Δ*sigL* mutant might in part be linked to disruption in the expression of particular σ^L dependent stress adaptation genes in

mutant cells. Such a hypothesis was investigated through transcript analysis of some genes with suggested roles in *L. monocytogenes* cold and NaCl salt stress tolerance, whose expression is partly influenced through σ^L dependent mechanisms. The levels of *oppA*, *cspD* and *clpP* transcripts were comparatively evaluated in the Δ*sigL* and wild type EGDe cells adapted to cold and NaCl stress conditions. To begin with the levels of *mptA* transcripts whose expression is known to be directly σ^L controlled were assessed in these two strains in order to confirm disruption of σ^L transcription control in the Δ*sigL* mutant strain. As expected *mptA* transcripts were found to be lower in Δ*sigL* compared to wild type EGDe cells. The *oppA* and *cspD* genes have been linked to experimentally confirmed cold adaptation roles in *L. monocytogenes* (Borezee *et al*., 2000; Schmid et al., 2009). Although there was no difference in *cspD* transcripts between the two strains, we found that *oppA* transcripts were significantly lower (11-fold) in Δ*sigL* compared to the wild type EGDe in cold adapted cells. Since the OppA protein is important for the function of the Opp permease, its reduced expression implies impaired functions of this oligopeptide transporter in cold adaptation of Δ*sigL* cells. On the other hand there were no significant ($p<0.05$) differences in thee *cspD* and *clpP* transcripts in NaCl stress adapted Δ*sigL* and EGDe cells. It thus seems that NaCl stress sensitivity observed in *L. monocytogenes* might not be associated with σ^L regulation of both *clpP* or *cspD* transcription.

Finally our study also further highlighted the influence of certain growth media conditions on the cold growth phenotypes associated with *L. monocytogenes*. Although clear cold growth defects were associated with Δ*sigL* EGDe strain in DM at 10 and 4℃, the same strain displayed minimal phenotypic differences from the wild type strain when evaluated in BHI cultures under same cold stress conditions. Similarly minimal cold growth phenotypes were observed when the growth of an *L. monocytogenes* 1043S *sigL* null mutant was compared to its parental wild type strain

in BHI at 4℃ (Chan et al., 2008). This is because *L. monocytogenes* growth under both osmotic and cold stress is enhanced when cryoprotective nutrients such as carnitine and betaine are present in the growth media (Wemekamp-Kamphuis et al., 2004, Angelidis et al., 2003). Similarly in this study carnitine or glycine betaine supplementation was found to reduce the cold stress sensitivity of the Δ*sigL* mutant strain in DM at 10℃. Cryoprotective nutrients, in particular carnitine, short peptides and amino acids are also abundant in BHI media. The presence of such nutrients in BHI may thus also mask cold sensitive phenotypes associated with inactivation of *L. monocytogenes* cold adaptation genes. Such media background influences should therefore be taken into consideration when deletion mutant strains of this organism are analyzed for their stress response phenotypes.

6. References

1. **Angelidis AS, and Smith GM**. Three transporters mediate uptake of glycine betaine and carnitine by *Listeria monocytogenes* in response to hyperosmotic stress. Appl. Environ. Microbiol. 2003; **69**:1013-1022.
2. **Arous S, Buchrieser C, Folio P, Glaser P, Namane A, Hebraud M, and Hechard Y.** Global analysis of gene expression in an *rpoN* mutant of *Listeria monocytogenes*. Microbiology. 2004a;**150**:1581-1590.
3. **Arous S, Dalet K, and Hechard Y.** Involvement of the *mpo* operon in resistance to class IIa bacteriocins in *Listeria monocytogenes*. FEMS Microbiol. Lett. 2004b;**238**:37-41.
4. **Becker LA, Cetin MS, Hutkins RW, and Benson AK.** Identification of the gene encoding the alternative sigma factor sigmaB from *Listeria monocytogenes* and its role in osmotolerance. J. Bacteriol. 1998;**180**:4547-4554.
5. **Becker LA, Evans SN, Hutkins RW, and Benson AK.** Role of sigma(B) in adaptation of *Listeria monocytogenes* to growth at low temperature. J. Bacteriol. 2000;**182**:7083-7087.
6. **Borezee E, Pellegrini E, and Berche P.** OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect. Immun. 2000;**68**:7069-7077.
7. **Buck M, Gallegos MT, Studholme DJ, Guo Y, and Gralla JD.** The bacterial enhancer-dependent sigma(54) (sigma(N)) transcription factor. J. Bacteriol. 2000;**182**:4129-4136.
8. **Bullock WO, Fernandez JM, and Short JM.** Xl-1 blue: A high efficiency plasmid transforming *recA Eshcerichia coli* strain with B-galactosidase selection. Biotechniques. 1987;**5**:376-379.

9. **Chan YC, Hu Y, Chaturongakul S, Files K D, Bowen BM, Boor KJ, and Wiedmann M.** Contributions of two-component regulatory systems, alternative sigma factors, and negative regulators to *Listeria monocytogenes* cold adaptation and cold growth. J. Food Prot. 2008;**71**:420-425.

10. **Chatterjee SS, Hossain H, Otten S, Kuenne C, Kuchmina K, Machata S, Domann E, Chakraborty T, and Hain T.** Intracellular gene expression profile of *Listeria monocytogenes*. Infect. Immun. 2006;**74**;1323-1338.

11. **Chaturongakul S, Raengpradub S, Wiedmann M, and Boor KJ**. Modulation of stress and virulence in *Listeria monocytogenes*. Trends Microbiol. 2008;**16**:388-396.

12. **Chen Y, Ross WH, Gray, MJ, Wiedmann M, Whiting RC, and Scott VN.** Attributing risk to *Listeria monocytogenes* subgroups: dose response in relation to genetic lineages. J. Food Prot. 2006;**69**:335-344.

13. **Ermolenko DN, and Makhatadze GI.** Bacterial cold-shock proteins. Cell Mol. Life Sci. 2002;**59**:1902-1913.

14. **Ferreira A, O'Byrne CP, and Boor KJ.** Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. Appl. Environ. Microbiol. 2001;**67**:4454-4457.

15. **Gaillot O, Pellegrini E, Bregenholt S, Nair S, and Berche P.** The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes*. Mol. Microbiol. 2000;**35**:1286-1294.

16. **Glaser P, Frangeul L, Buchrieser C, et al.** Comparative genomics of *Listeria* species. Science. 2001;**294**:849-852.

17. **Gray MJ, Zadoks RN, Fortes ED, et al**. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. Appl. Environ. Microbiol. 2004;**70**:5833-5841.

18. **Horton RM, Cai ZL, Ho SN, and Pease LR.** Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques. 1990;**8**:528-535.

19. **Lianou A, and Sofos JN.** A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. J. Food Prot. 2007;**70**:2172-2198.

20. **Liu S, Graham JE, Bigelow L, Morse PD, and Wilkinson, BJ.** Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. *Appl. Environ. Microbiol.* 2002;**68**:1697-1705.

21. **Metzger R, Brown DP, Grealish P, Staver MJ, Versalovic J, Lupski JR, and Katz L.** Characterization of the macromolecular synthesis (MMS) operon from *Listeria monocytogenes*. Gene 1994;**151**:161-166.

22. **Monk IR, Gahan CG, and Hill C.** Tools for functional postgenomic analysis of *listeria monocytogenes*. Appl. Environ. Microbiol. 2008;**74**:3921-3934.

23. **Moorhead SM, and Dykes GA.** The role of the sigB gene in the general stress response of *Listeria monocytogenes* varies between a strain of serotype 1/2a and a strain of serotype 4c. Curr. Microbiol. 2003;**46**:461-466.

24. **Nufer U, Stephan R, and Tasara T.** Growth characteristics of *Listeria monocytogenes, Listeria welshimeri* and *Listeria innocua* strains in broth cultures and a sliced bologna-type product at 4 and 7 degrees C. Food Microbiol. 2007;**24**:444-451.

25. **Okada Y, Okada N, Makino S, Asakura H, Yamamoto S, and Igimi S.** The sigma factor RpoN (sigma54) is involved in osmotolerance in *Listeria monocytogenes*. FEMS Microbiol. Lett. 2006;**263**:54-60.

26. **Pal, A, Labuza TP, and Diez-Gonzalez F.** Evaluating the growth of *Listeria monocytogenes* in refrigerated ready-to-eat frankfurters: influence of strain,

temperature, packaging, lactate and diacetate, and background microflora. J. Food Prot. 2008;**71**:1806-1816.

27. **Phan-Thanh L, and Mahouin F.** A proteomic approach to study the acid response in *Listeria monocytogenes*. Electrophoresis. 1999;**20**:2214-2224.

28. **Premaratne RJ, Lin WJ, and Johnson EA.** Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. 1991;**57**:3046-3048.

29. **Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MU, Dharsana KS, Prasad SP, and Vijila HM.** Listeria--review of epidemiology and pathogenesis. J. Microbiol. Immunol. Infect. 2007;**40**:4-13.

30. **Rea RB, Gahan CG, and Hill C.** Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. Infect. Immun. 2004;**72**:717-727.

31. **Robichon D, Gouin E, Debarbouille M, Cossart P, Cenatiempo Y, and Hechard Y.** The rpoN (sigma54) gene from *Listeria monocytogenes* is involved in resistance to mesentericin Y105, an antibacterial peptide from *Leuconostoc mesenteroides*. J. Bacteriol. 1997;**179**:7591-7594.

32. **Schmid B, Klumpp J, Raimann E, Loessner MJ, Stephan R, and Tasara T.** Role of cold chock proteins (Csp) for growth of *Listeria monocytogenes* under cold and osmotic stress conditions. 2009; in press.

33. **Sheng Y, Mancino V, and Birren B.** Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucleic Acids Res. 1995;**23**:1990-1996.

34. **Smith K, and Youngman P.** Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis* spoIIM gene. Biochimie 1992;**74**:705-711.

35. **Swaminathan B, and Gerner-Smidt P.** The epidemiology of human listeriosis. Microbes Infect. 2007;**9**:1236-1243.

36. **Tasara T, and Stephan R**. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. FEMS Microbiol. Lett. 2007;**269**:265-72.

37. **Wemekamp-Kamphuis HH, Sleator RD, Wouters JA, Hill C, and Abee T.** Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of *Listeria monocytogene*s at low temperatures. Appl. Environ. Microbiol. 2004;**70**:2912-2918.

38. **Wiegeshoff F, Beckering CL, Debarbouille M, and Marahiel MA.** Sigma L is important for cold shock adaptation of *Bacillus subtilis*. J. Bacteriol. 2006;**188**:3130-3133.

39. **Zhang C, Nietfeldt J, Zhang M, and Benson AK.** Functional consequences of genome evolution in *Listeria monocytogenes*: the *lmo0423* and *lmo0422* genes encode sigmaC and LstR, a lineage II-specific heat shock system. J. Bacteriol. 2005;**187**:7243-7253.

40. **Zhu K, Bayles DO, Xiong A, Jayaswal RK, and Wilkinson BJ.** Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain alpha-keto acid dehydrogenase. Microbiol. 2005;**151**:615-623.

7. Tables

Table 1. Bacterial strains, plasmids and oligonucleotides

Materials	Description	References
Bacteria		
L. monocytogenes EGDe	Genome sequenced wild type 1/2a strain	Glaser *et al.,* 2001
EGD-e Δ*sigL*	1290 bp in-frame deletion of the *sigL* gene	This study
E. coli XL-1 Blue	Wild type laboratory strain for routine plasmid propagation and cloning applications	Bullock *et al.,* 1987
Plasmids		
pKSV7	Temperature sensitive Gram positive bacteria integrational vector	Smith &Youngman 1992
pKVS7-Δ*sigL*	Temperature sensitive Gram positive integrational vector: Δ*sigL* deletion construct	This study
Oligonucleotides		
	SOE PCR primers	
SOE-P1-*rpoN*-A[a]	CG*GGATCC*ACCGCGCAGGACGCGT	This study
SOE-P1-*rpoN*-B[c]	CGTTTAGAATCTAATAATATTCCTTCTTCCTCTAAAAGAAAA AGAT	This study
SOE-P1-*rpoN*-C[c]	AGGAAGAAGGATATTATTAGATTCTAAACG CACATTAAAACCTC	This study
SOE-P1-*rpoN*-D[b]	*GGAATTC*CAGGACTAACTCGCTTCGGA	This study
	Realtime PCR primers	
16S rRNA-fw	CTTCCGCAATGGACGAAAGT	This study
16S rRNA-rv	CTCATCGTTTACGGCGTG	This study
rpoN-fw	TTGAAAGATGTTAGTGCGG	This study
rpoN-rv	CGGACTCAAACTCAGAA	This study
oppA-fw	CAGCGGATTCAGCTCT	This study
oppA-rv	ACGGATAATGCACTATAA	This study
trxB-fw	TAATGATTGAACGCGGTG	This study
trxB-rv	TGTAGGTTTTCGACCCAG	This study
clpP-fw	CAAACTAGCCGCGGTG	This study
clpP-rv	CGCCAAGTGGTTGGTGA	This study

[a]The *Eco*RI recognition sequence incorporated in primer to facilitate cloning is in italics

[b]The *Bam*HI recognition sequence incorporated in primer to facilitate cloning is in italics

[c]The complementary overhang region in SOE-P1-SigL-B and, SOE-P1-SigL-C SOE PCR primers are underlined

8. Figure legends

Figure 1. *sigL* gene expression associated with exposure of wild-type *L. monocytogenes* EGDe cells to cold (10°C), NaCl (BHI plus 3% NaCl) and acid (BHI adjusted to pH 6.0 with lactic acid) stress conditions. In all cases *sigL* gene expression of mid exponential growth phase stage EGDe cells was determined. Normalized log-transformed levels of *sigL* transcripts of mid exponential growth phase EGDe cells adapted to different conditions that included (A) BHI 37°C (control), (B) BHI 10°C (cold stress), BHI plus 3% NaCl (NaCl stress) and BHI adjusted to pH 6.0 using lactic acid (acid stress). The results presented reflect the mean and standard deviation (error bars) of three independent experimental runs. Statistically significant ($P<0.05$) differences in *sigL* mRNA transcript levels between cold, NaCl and acid stress exposed, and the non-stress exposed control EGDe cells grown in BHI at 37°C, are highlighted with an asterix.

Figure 2. Growth of wild-type EGDe and its Δ*sigL* derivative at 10°C in (A) BHI and (B) DM; (C) Growth of the Δ*sigL* mutant in DM at 10°C with or without supplementation of 1mM glycine betaine or carnitine. The presented results (log CFU/ml) reflect the mean and standard deviation (error bars) of three independent experimental runs.

Figure 3. (A) Growth of wild-type EGDe and Δ*sigL* mutant in DMS (DM plus 2.2% NaCl) at 37°C; (B) Growth of the Δ*sigL* mutant in DM and DMS at 10°C. The presented results reflect the mean and standard deviation (error bars) of three independent experimental runs.

Figure 4. Growth of wild type EGDe and Δ*sigL* strains at 4℃ in (A) regular BHI (pH 7.4) and (B) acidified BHI (pH 6.0). The presented results reflect the mean and standard deviation (error bars) of three independent experimental runs.

Figure 5. Transcriptional analysis of σ^L dependent stress adaptation genes involved in cold and NaCl stress tolerance. Normalized log-transformed levels of *(A) mptA*, *oppA* and *cspD* transcripts in exponential growth phase wild-type EGDe and ΔsigL cells adapted to cold stress (BHI at 10℃) conditions; (B) *(A) mptA*, *clpP* and *cspD* transcripts in exponential growth phase wild-type EGDe and ΔsigL adapted to NaCl stress (BHI plus 3%NaCl) conditions. These results reflect the mean and standard deviation (error bars) of three separate experimental runs. Statistically significant (P<0.05) differences in *sigL* mRNA transcript levels between wild-type EGDe and D*sigL* mutant cells are denoted by an asterix.

9. Figures

Figure 1

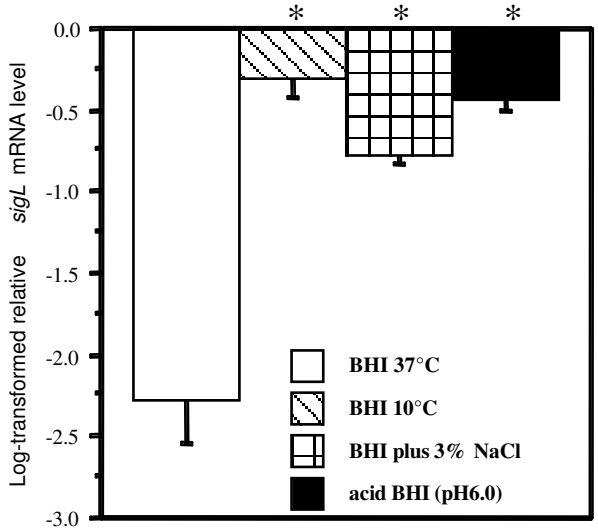


Figure 2

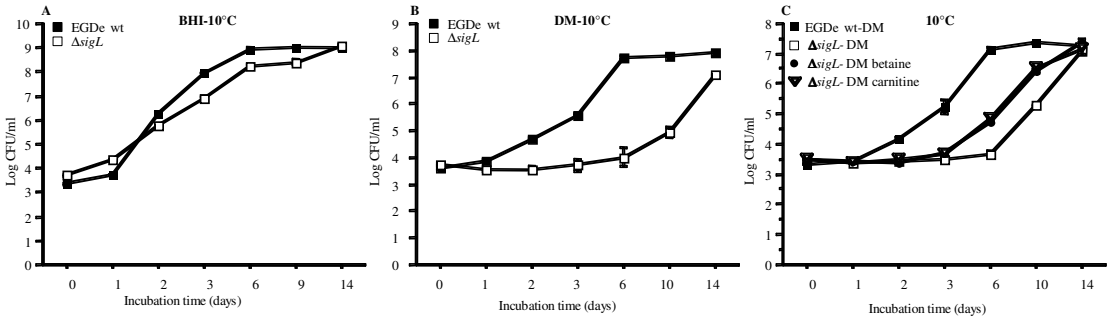


Figure 3

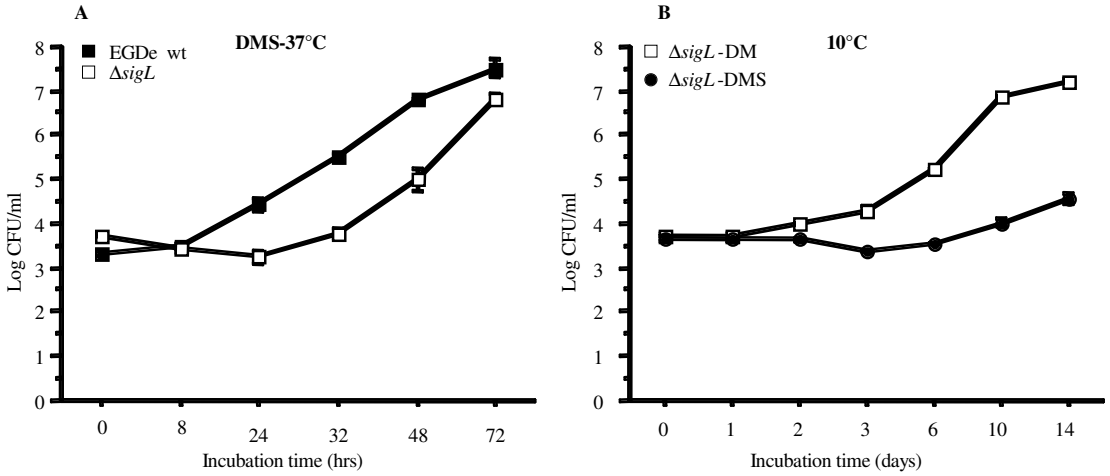


Figure 4

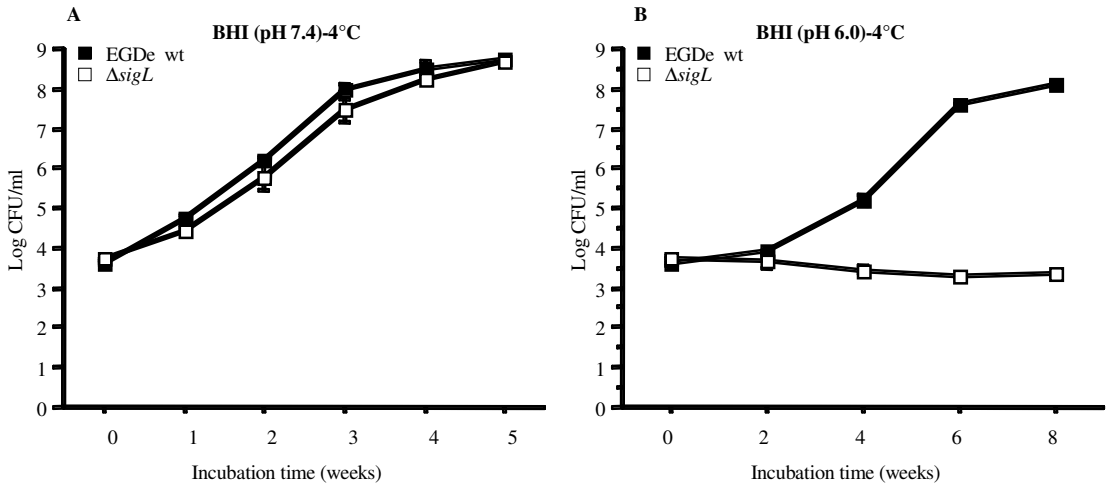
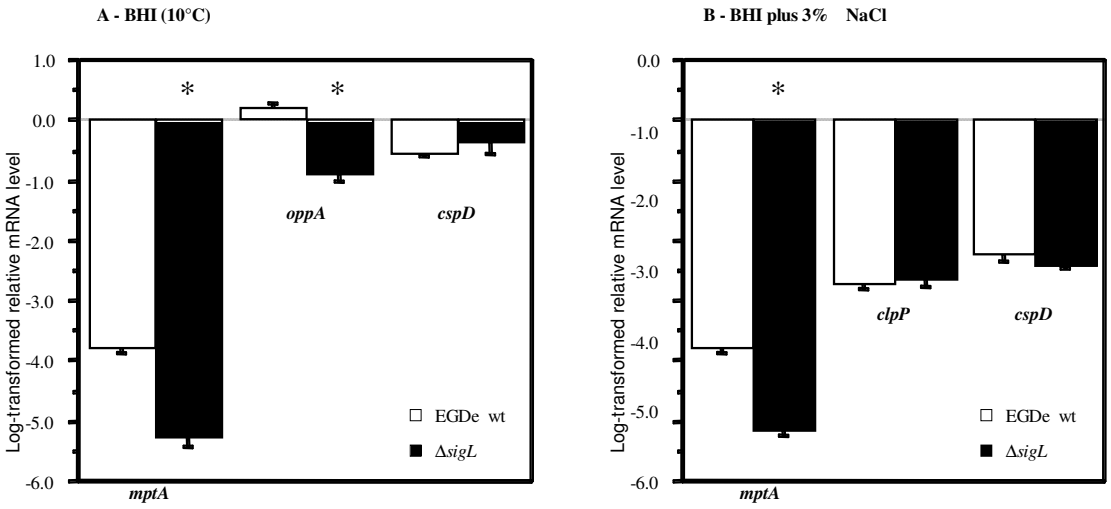


Figure 5



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